Computational Identification of Small Molecule Inhibitors to Target ROR1 Protein

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Certificate

I hereby declare that the Short Synopsis titled "Computational Identification of Small Molecule Inhibitors to Target ROR1 Protein" being submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy in the Department of Biotechnology under the School of Engineering and Technology of Manav Rachna International Institute of Research and Studies, Faridabad during the academic year 2024. It is a bona fide record of original work carried out under the supervision of Dr. Rashmi Rameshwari and Dr. Indrakant Kumar Singh and has not been submitted in full or part to any other university or institute for a degree or diploma. Further, it is certified that Short Synopsis is plagiarism free and proof has been submitted.

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Abstract

The ROR1 protein has emerged as a highly promising therapeutic target due to its involvement in critical signaling pathways that is linked to severe pathological conditions. Current treatment strategies, including monoclonal antibodies, antibody-drug conjugates, and immunotoxins, have demonstrated considerable potential in targeting ROR1, but their clinical efficacy is often limited by significant drawbacks, such as immune-related adverse effects, off-target toxicity, and the eventual emergence of therapeutic resistance. To address these challenges and improve therapeutic outcomes, this study introduces a comprehensive *in-silico* research methodology that employs advanced computational techniques, including shape screening, well-tempered metadynamics, and alchemical binding free energy calculations. These approaches are applied to systematically identify, evaluate, and optimize lead compounds with a strong and stable binding affinity for the pseudokinase domain of ROR1 protein. By focusing on this domain, the study aims to discover novel, ROR1 specific inhibitors that offer enhanced efficacy, improved safety profiles, and a reduced risk of resistance development, thereby significantly contributing to the advancement of next-generation, targeted therapies for ROR1 associated diseases.

Keywords: Cancer, Drug Discovery, Inhibitor, In-silico, Kinase, Pseudokinases, ROR1, Small Molecules

List of Abbreviations

1. ADC - Antibody-Drug Conjugate
2. ABFEP - Absolute Binding Free Energy Perturbation
3. AKT - Protein Kinase B
4. ATP - Adenosine Triphosphate
5. CAR T - Chimeric Antigen Receptor T-cell
6. CNS - Central Nervous System
7. CRD - Cysteine-rich Domain
8. CREB - cAMP Response Element-Binding protein
9. CTLA-4 - Cytotoxic T-Lymphocyte Associated Protein 4
10. DFE - Dissociation Free Energy
11. EGFR - Epidermal Growth Factor Receptor
12. ERK - Extracellular Signal-Regulated Kinase
13. HIF-1 - Hypoxia Inducible Factor 1
14. HER2 - Human Epidermal Growth Factor Receptor 2
15. HSP90 - Heat Shock Protein 90
16. HTVS - High Throughput Virtual Screening
17. IG - Immunoglobulin-like
18. JAK2 - Janus Kinase 2
19. KD - Kringle Domain
20. MAPK - Mitogen-activated Protein Kinase
21. mAbs - Monoclonal antibodies
22. MD - Molecular Dynamics
23. mTOR - Mammalian Target of Rapamycin
24. MMGBSA - Molecular Mechanics Generalized Born Surface Area
25. NF-kB - Nuclear Factor kappa-light-chain-enhancer of activated B cells
26. NGS - Next-Generation Sequencing
27. ns - nanoseconds
28. PD-1 - Programmed Cell Death Protein 1
29. PD-L1 - Programmed Death-Ligand 1
30. PDB - Protein Data Bank
31. PI3K - Phosphoinositide 3-kinase

32. PNS - Peripheral Nervous System
33. PRD - Proline-rich Domain
34. RTK - Receptor Tyrosine Kinase
35. SP - Standard Precision
36. STAT3 - Signal Transducer and Activator of Transcription 3
37. TI - Thermodynamic Integration
38. TKIs - Tyrosine Kinase Inhibitors
39. Wnt - Wingless-related integration site
40. XP - eXtra Precision

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1. Description of Broad Area

One in every five individuals develops cancer in their lifetime, with men facing a risk of approximately one in nine and women facing one in twelve chances of succumbing to this disease. By 2050, over 35 million new cases will be reported globally, creating a health crisis (Bray et al., 2024). The treatment landscape has been experiencing significant shifts in the fight against cancer, marked by notable advancements such as gene therapy, immunotherapy, and targeted therapy. Gene therapy entails using therapeutic genes to either directly eliminate malignant cells or modify flawed genetics responsible for cancer. Although still in its early stages, its potential is immense (Cesur-Ergun & Demir-Dora, 2023; Weichselbaum & Kufe, 1997). Immunotherapy operates by activating the body's immune system to identify and eliminate tumors effectively, employing biological substances from living organisms (Institute, 2019a). Targeted therapies have emerged as foundational elements of precision medicine, targeting various malignancies causing proteins that regulate the cell division process and are linked to metastasis development (Institute, 2022b; Zhong et al., 2021).

One of the most targeted protein families is kinase. The members are essential in cellular processes, regulating signaling pathways critical for growth, differentiation, and apoptosis. Upon dysregulation, kinases promote uncontrolled proliferation, angiogenesis, and metastasis, making them suitable targets (Cohen et al., 2021; Kennedy & Hobbs, 2018; Plati et al., 2008). Despite the success in targeting kinases, prominent issues like toxicity, signaling network redundancies, development of resistance, and off-target effects regularly surface and heavily impact the normal functioning of the body (Barouch-Bentov & Sauer, 2011; Bhullar et al., 2018).

Targeting kinases is a double-edged sword. Therefore, there is a need to find proteins that are not as important for the body's normal functioning as for the cancer cells to survive and grow. Pseudokinases are one such group of proteins that share structural similarity with kinases and have low to no kinase activity but support cancer cells to survive and grow (Sheetz & Lemmon, 2022; Sheetz et al., 2020). Therefore, the vision of the current work is to utilize the advantages of targeted therapy to find small-molecule inhibitors that can alter the function of pseudokinases of our interest.

1.1. Targeting Pseudokinases:

About ten percent of the human kinome consists of pseudokinases, underscoring their importance (Kwon et al., 2019). The term "pseudokinase" suggests a structure similar to kinases but lacks crucial conserved catalytic sites necessary for kinase activity (Boudeau et al., 2006; Manning et al., 2002). However, research has revealed exceptions to this notion. WNK1 is categorized as a pseudokinase yet exhibits slower but catalytic activity (Sengupta et al., 2012; Xu et al., 2000). Additionally, the

receptor guanylyl cyclase (RGC) family includes pseudokinase members only (Edmund et al., 2019). Mutations in the pseudokinase domains of proteins like JAK2 and ErbB3 are implicated in myeloproliferative neoplasms and gastrointestinal cancers, respectively (Jaiswal et al., 2013; Jones et al., 2005). Small molecules like APS-2-79 bind to the ATP binding site of the KSR2 pseudokinase domain, suppressing ERK signaling and enhancing the efficacy of MEK inhibitors (Dhawan et al., 2016). Deucravacitinib, a compound specifically targeting the TYK2 JH2 pseudokinase domain, has entered clinical trials for several diseases, including systemic lupus erythematosus, ulcerative colitis, Crohn's disease, and psoriasis (Papp et al., 2018). Many pseudokinases, such as Receptor Tyrosine Kinase like Orphan Receptor 1 or RTKs like Orphan Receptor (ROR1), remain to target.

1.2. ROR1 a Potential Target:

ROR1 is an oncofetal receptor that belongs to the cell surface Receptor Tyrosine Kinase (RTK) family (Zheng et al., 2016). It is expressed at high levels during early embryonic development, but its expression diminishes significantly around day 16, resulting in very low levels in adult tissues (Cruz, 2023a; Fagerberg et al., 2014). Its limited expression in adults is restricted to the heart, lungs, kidneys, skeletal muscles, fat tissues, digestive system, some endocrine glands, and immature B cells, where its function is yet unknown (Balakrishnan et al., 2017). Like embryonic cells, its expression increases in several cancers, mainly originating from neuroectodermal tissues of the CNS or peripheral nervous system (PNS) (Kipps, 2022; Saleh et al., 2019).

'Orphan receptor' refers to receptors whose endogenous ligands have not been fully characterized or identified (Siepe et al., 2022). Despite being termed as orphan receptors, ROR1 and its closely related homolog, Receptor tyrosine kinase-like Orphan Receptor 2 (ROR2), have been found to bind to various ligands, including Wnt5a and Wnt5b, which regulate diverse cellular processes during development and tissue homeostasis (Kipps, 2022; Parker et al., 2023; Rebagay et al., 2012).

ROR1 is not intrinsically catalytically active, but it does play a significant role in the progression, survival, and proliferation of aggressive cancer via activating pathways such as EGFR-mediated, Wnt signaling, and PI3K/AKT/mTOR. Increased tumor size, lymphatic metastasis, and TNM stage (II and IV) are all correlated with ROR1 upregulation. In several malignancies, knockout experiments showed reduced cell proliferation and higher apoptosis, suggesting ROR1 as a possible target for therapy (Kipps, 2022; Saleh et al., 2019). After examining its structure, scientists found that it has a binding site, and by targeting it, ROR1's downstream signaling and function can be altered in our favor (Sheetz et al., 2020).

1.3. Structure of ROR1:

ROR1 is a glycosylated type I membrane protein member of the ROR subfamily within the RTK family. In humans, it is found on chromosome 1p31.3. Two transcript variants are produced by alternative splicing of the ROR1 gene. A protein with 937 amino acids is encoded by transcript variant 1, which has 9 exons. In contrast, a shorter isoform with 393 amino acids is encoded by transcript variant 2, which has 7 exons. The locations of the coding area for variant 1 and variant 2 are hg38 chr1:63,774,017-64,181,498 and hg38 chr1:63,774,418-64,142,658, respectively (Cruz, 2023a, 2023b) (Figure 1: A&B).

Structurally, ROR1 comprises multiple domains essential for its function. The extracellular domain at N-terminus is involved in ligand binding, which triggers downstream signaling events. This domain contains immunoglobulin-like (IG), cysteine-rich (CRD), and Kringle domain (KD) that facilitate protein-protein interactions. The transmembrane domain anchors ROR1 to the cell membrane, while the juxtamembrane section promotes its movement into the nucleus, acting as a transcription factor influencing the cytoskeleton network and cell migration. The intracellular (cytoplasmic) domain harbors the tyrosine kinase-like domain devoid of key catalytic residues, serine/threonine-rich, and proline-rich domains (PRD). These domains are responsible for downstream signaling upon ligand binding (Borcherding et al., 2014; Zhao et al., 2021) (Figure 1: C&D)

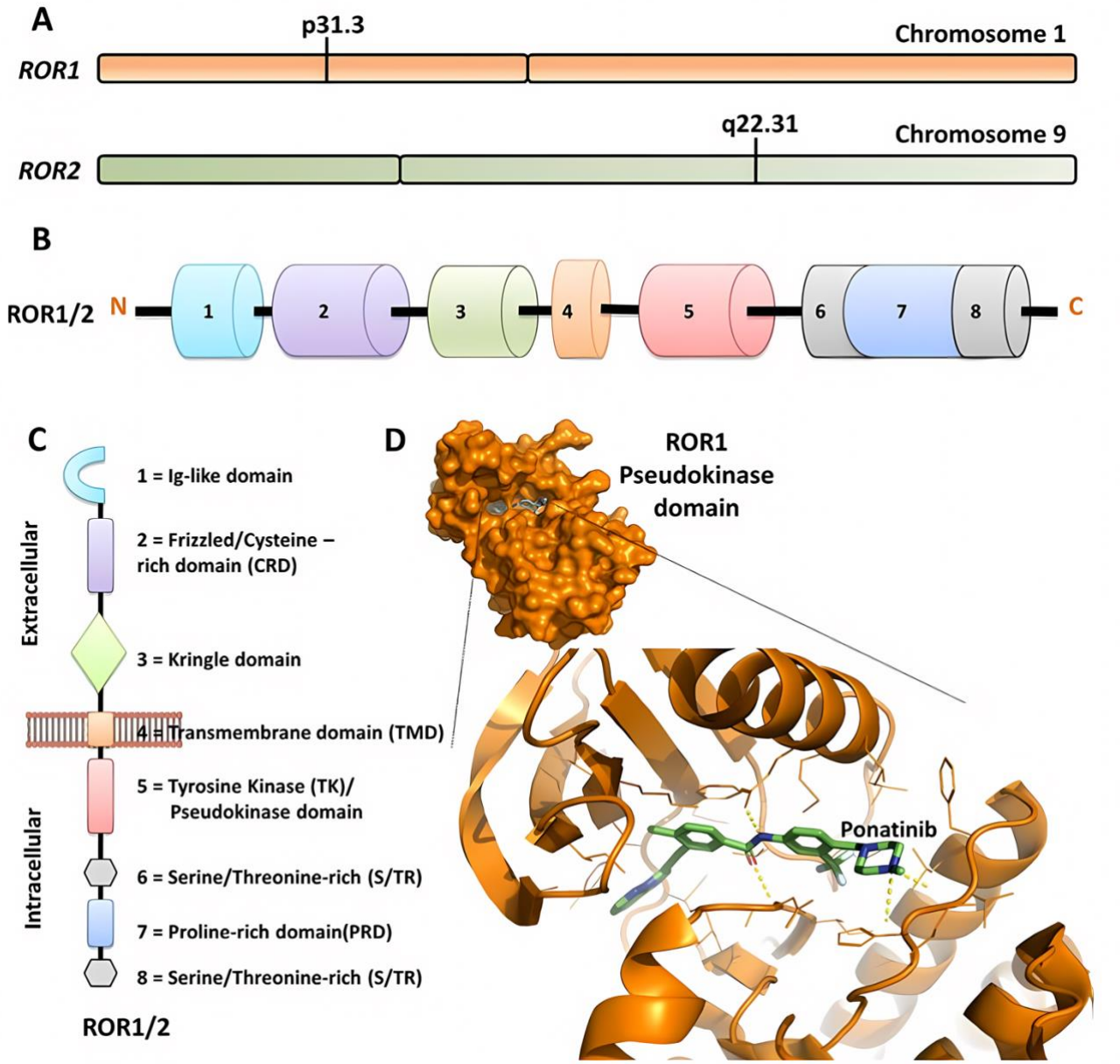

Figure 1: A. Localization of ROR1 and ROR2 on the human chromosome; B. Illustration of distinct domains, with the extracellular domain situated at the N-terminal and the intracellular domain positioned at the C-terminal; C. Schematic representation of various ROR1 domains. The pseudokinase domain is a small molecule targetable site situated intracellularly; D. The molecular structure of the pseudokinase domain is bound to Ponatinib within the binding site. The structural visualization is made by using the PDB ID 6TU9. Source: (Rustagi et al., 2022)

1.4. Pathways Associated with ROR1:

Wnt5a stimulates ROR1 to initiate the signaling. When Wnt5a comes and attaches to the CRD domain of the ROR1 present outside the cell, it triggers ROR1 to form a heterodimer with ROR2, but the process is not required for ROR1 to initiate multiple signaling pathways (involved pathways are depicted in Figure 2). When ROR2's intracellular domain interacts with the intracellular domain of ROR1, it activates ARHGEF1/2/6, activating RhoA and Rac1, thereby promoting the proliferation and migration of cancer cells (Yu et al., 2016). Similarly, 14-3-3ζ interacts with the C-terminal serine/threonine domain of ROR1, activating ARHGEF2 and fostering proliferation and migration (Yu et al., 2017). DOCK2's interaction with ROR1's PRD induces the MAPK pathway and ERK1/2 activation, resulting in the transcription of factors like CREB and HIF-1, crucial for the proliferation and survival of cancer cells (M. K. Hasan et al., 2021; Md Kamrul Hasan et al., 2021). Additionally, HS1/cortactin interacts with ROR1's PRD, activating ARHGEF1 and influencing planar cell polarity, filopodia formation, and chemokine-directed migration (Hasan et al., 2019; Hasan et al., 2017). PI3K's interaction with ROR1 activates the PI3K/AKT/mTOR pathway and NRF2, which is crucial for survival, anti-apoptotic response, and resistance to therapy (Daneshmanesh et al., 2013; Sanchez-Lopez et al., 2020).

Further, the interaction with PI3K activates NF-kB, which further activates the JAK2/STAT3 pathway, providing resistance to therapy and generating anti-apoptotic responses (Guo et al., 2022; Rozovski et al., 2019). Lyn's interaction with ROR1's TKD induces phosphorylation at tyrosine residues (Y645 and Y646), impacting surface ROR1 dynamics during chemotaxis (Dave et al., 2022). Lastly, HSP90 interacts with the ELHHPNIV (residues from 533 to 540) binding motif of ROR1, initiating the formation of the αC-β4 loop that stabilizes ROR1, protecting it from proteasomal degradation, thus contributing to pathway integrity (Liu et al., 2020). Overall, these interactions orchestrate a network that regulates diverse cellular processes, from proliferation and migration to survival and chemotaxis, highlighting the multifaceted role of ROR1 signaling in cellular physiology and cancer (Kipps, 2022; Mouawad et al., 2024).

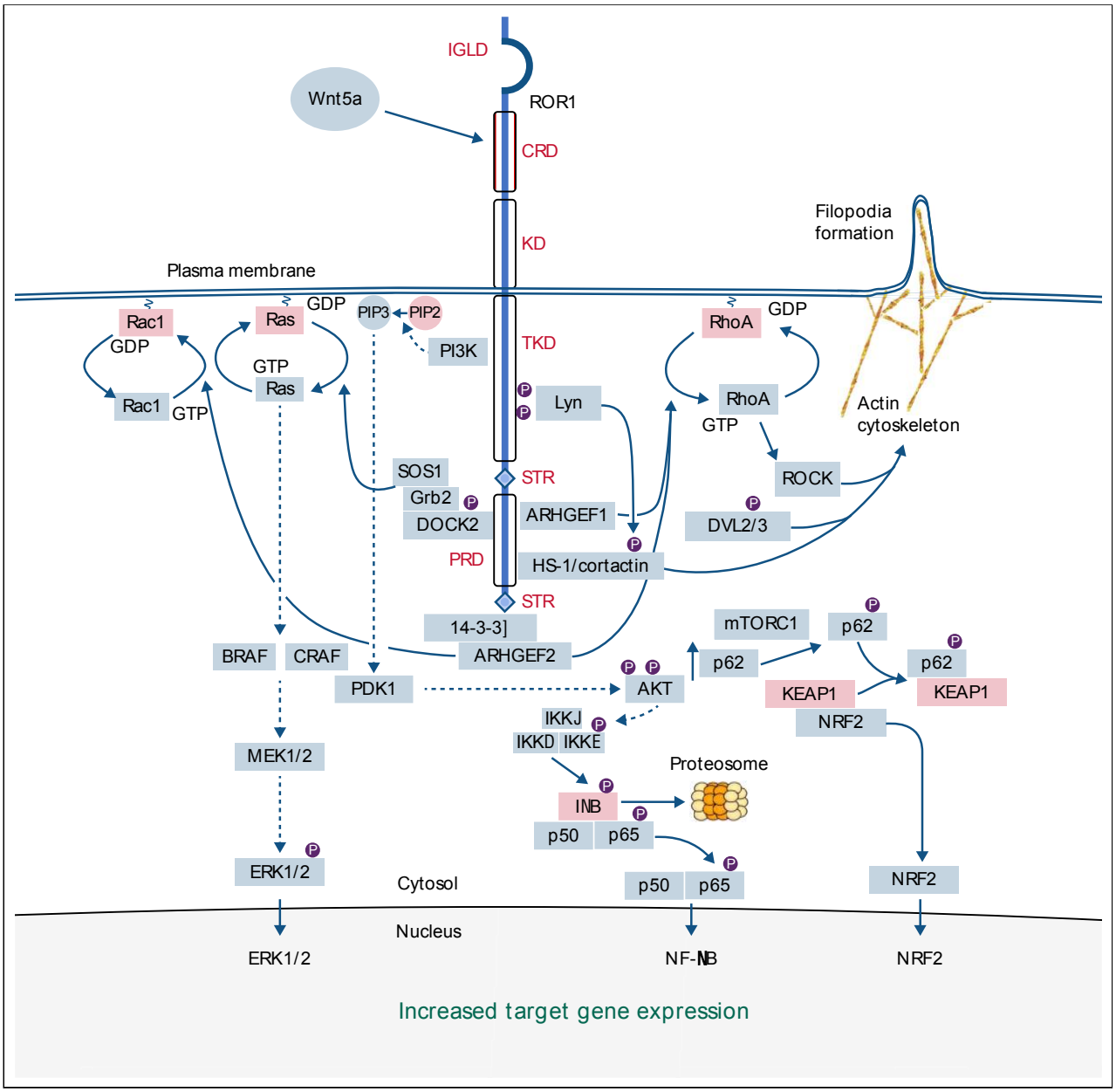

Figure 2: Structural depiction of ROR1 showcasing the involvement of its domains in diverse pathways. (Kipps, 2022)

1.5. Current Ways to Target ROR1:

ROR1-targeting therapeutics are developing and providing a wide range of therapy options (therapeutics and their target site is depicted in Figure 3). Among these, anti-ROR1 Monoclonal Antibodies (mAbs) such as 3B8 targeting the IG domain, 1C11, and 1D8 focusing on the CRD, 2A2, and 2D11 against N-terminal epitopes and 4C10 and 4A7 honing in on the KD domain. These antibodies hold promise in addressing chronic lymphocytic leukemia and mantle cell lymphoma (Mouawad et al., 2024). Amongst the many mAbs, Zilovertamab (UC-961) showcases internalization

capabilities and a knack for inhibiting Wnt5a-induced survival impacts on leukemic cells. Its Phase 1 trials in chronic lymphocytic leukemia patients underscore its potential in clinical settings (Shatsky et al., 2024).

Beyond antibodies, there are other types of targeted medicines available. For example, Zilovertamab Vedotin is an antibody-drug combination (ADC) that targets cell division and induces apoptosis in cells, which is particularly effective against Richter syndrome (Ozcan et al., 2023; Wang et al., 2022). Similarly, in ROR1-positive mantle cell lymphoma, BT-1 immunotoxin preferentially triggers apoptosis (Baskar et al., 2012). Furthermore, 2A2-miR-29b-ILP introduces immunoliposome-encapsulated regulatory RNA, which exhibits strong anti-leukemic efficacy against ROR1-expressing chronic lymphocytic leukemia cells. An OSU-2S sphingosine analog is delivered by 2A2-OSU-2S-ILP, which causes apoptosis in mantle cell lymphoma and chronic lymphocytic leukemia. A bispecific antibody called NVG-111, on the other hand, shows promise in directing ROR1-positive malignant cells and stimulating cytotoxic T cells, providing hope for solid tumors as well as chronic lymphocytic leukemia (Mouawad et al., 2024).

Turning to small molecule inhibitors, KAN0173631, KAN0438063, KAN0438175-T, KAN0439834, and KAN0441571-C exhibit efficacy in inducing apoptosis and dephosphorylating ROR1 in chronic lymphocytic leukemia and diffuse large B-cell lymphoma (Daneshmanesh et al., 2018; Ghaderi et al., 2020; Ghaderi et al., 2023; Ghaderi et al., 2021; Ghaderi et al., 2022; Wang et al., 2021). Additionally, through distinct pathways, ARI-1 impacts non-small cell lung cancer (Liu et al., 2019) whereas Strictinin and CID1261330 impact triple-negative breast cancer (Fultang et al., 2019; Gupta et al., 2023). CSF1R-IN-1 and MCULE-8715589337 were tested on pancreatic cancer cells (Singh et al., 2024). Naturally occurring compounds like DB03208 contribute to this pharmacological tapestry (Nath et al., 2017).

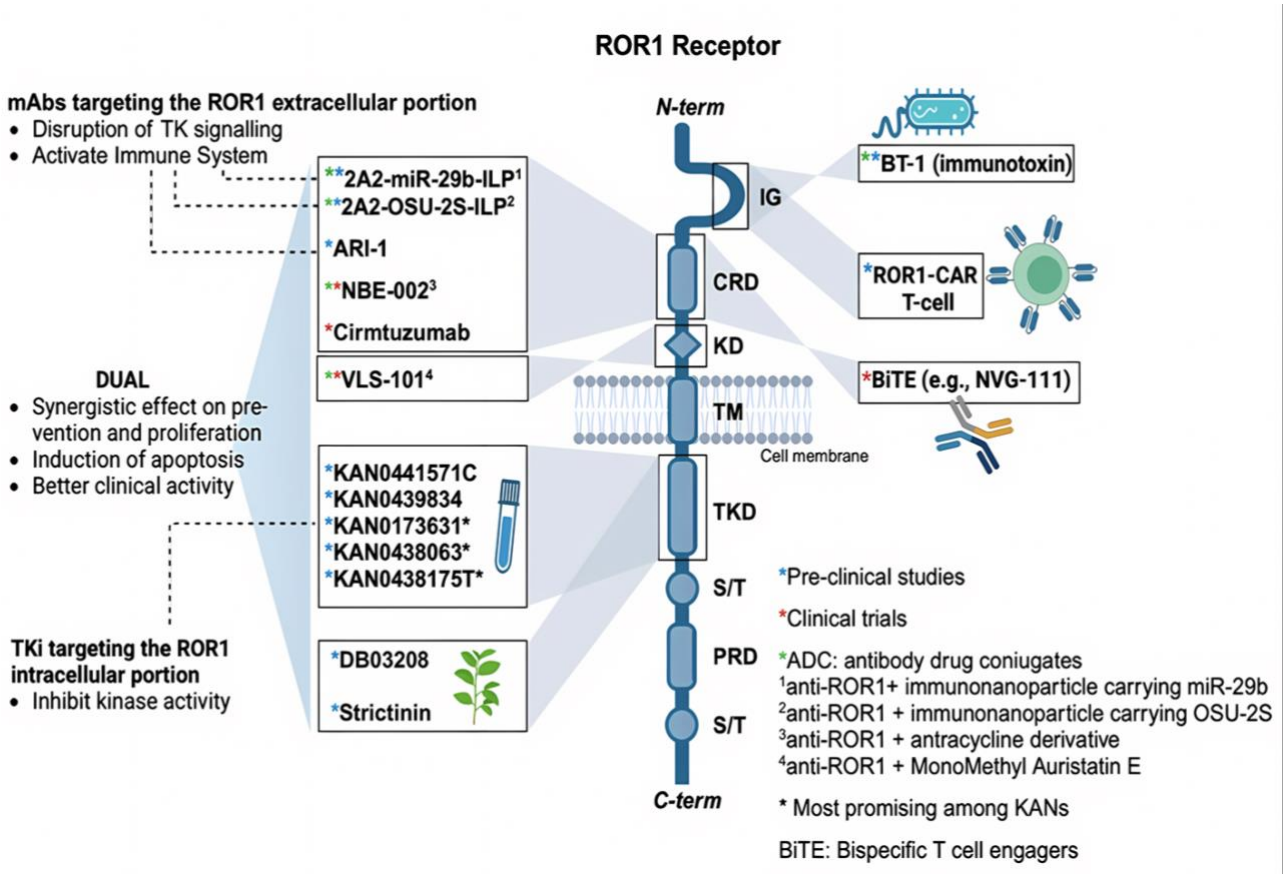

Figure 3: Various therapeutics altering the function of the ROR1 protein, each targeting specific domains. (Mouawad et al., 2024)

1.6. Limitations of Current Therapies

Each therapy described above exhibits potential limitations that warrant consideration in their clinical application. Anti-ROR1 mAbs face challenges in patients with tumors lacking ROR1 expression and could induce immune-related adverse effects with prolonged use. Zilovertamab, while effective in inhibiting Wnt5a-induced survival impacts, may encounter resistance mechanisms and pose risks of infusion reactions or off-target toxicities. Similarly, Zilovertamab Vedotin's efficacy could be hampered by systemic toxicity and resistance development (Descotes, 2009; Hansel et al., 2010; Kumar et al., 2020; Osorio-Rodriguez et al., 2023). BT-1 immunotoxin's efficacy may be limited by tumor heterogeneity and immunogenicity (Lim & Ma, 2019). Delivery challenges and off-target effects may constrain the effectiveness of 2A2-miR-29b-ILP, while systemic toxicity and variable ROR1 expression levels could impede 2A2-OSU-2S-ILP. NVG-111's efficacy might be compromised by T cell activation-related adverse effects and limitations in solid tumor penetration (Segal & Slack, 2020). Despite their specificity, small molecule inhibitors may face drug resistance issues, off-target effects, and limited tumor tissue penetration. DB03208's lack of specificity and

potential adverse effects on normal cellular functions further highlight the need for ongoing refinement and optimization efforts across these therapeutic modalities (Liu et al., 2022; Zhong et al., 2021).

2. Literature Review

Approximately 20 million new cancer diagnoses and 9.7 million fatalities occur worldwide. Statistics reveal that one in every five individuals will develop cancer in their lifetime, with men facing a risk of approximately one in nine and women facing about a one-in-twelve chance of succumbing to this disease. Projections suggest that by 2050, over 35 million new cases may be reported globally, creating a health crisis (Bray et al., 2024). Prominent contributors to the trend are aging populations, lifestyle choices such as smoking, obesity, poor circadian cycle, and pollution (Macek et al., 2020; Schottenfeld et al., 2013). Regarding specific types of cancers prevalent among genders - prostate, lung, and colorectal are more commonly found among males. In contrast, breast and cervical cancers remain at the forefront when it comes to affecting females (Bray et al., 2024).

The cancer treatment landscape experiences significant shifts marked by notable advancements such as gene therapy, immunotherapy, and targeted therapy. Gene therapy uses therapeutic genes to either directly eliminate malignant cells or modify flawed genetics responsible for cancer (Cesur-Ergun & Demir-Dora, 2023; Weichselbaum & Kufe, 1997). Although still in its early stages, its potential is immense. Immunotherapy employs biological substances from living organisms to activate the body's immune system and eliminate tumors effectively (Institute, 2019a). It involves immune checkpoint inhibitors, CAR T Cell Therapy, Monoclonal antibodies, vaccines, and Immune system modulators (Gupta et al., 2022; Institute, 2019a). Targeted therapies emerge as foundational elements of precision medicine, targeting various malignancy-causing proteins that regulate the cell division process linked to metastasis development (Institute, 2022b). It involves small-molecule drugs and Monoclonal antibodies (Min & Lee, 2022; Padma, 2015; Sun et al., 2021). The small molecule drugs are targeted to bind to the specific protein by easily passing the cell membrane (Zhong et al., 2021), (Cesur-Ergun & Demir-Dora, 2023; Weichselbaum & Kufe, 1997) (Institute, 2019a) (Institute, 2022b)

T cells, which are immune cells, recognize and bind to partner proteins on other cells, including specific tumor cells, and immunological checkpoints are activated. It functions by preventing partner proteins from binding with checkpoint proteins. As a result, the "off" signal is not sent, enabling the T cells to kill cancer cells (Banday & Abdalla, 2023; Sharma et al., 2017). Immune checkpoint inhibitors for the treatment of different cancers have been approved. These inhibitors target PD-1,

PD-L1, and CTLA-4 (Shiravand et al., 2022; Wojtukiewicz et al., 2021). CAR T Cell Therapy uses patient-derived T cells that have undergone genetic modification in a lab to improve their ability to identify and eliminate cancer cells (Institute, 2022a; Rohit Reddy et al., 2021). Monoclonal antibodies are used in targeted and immune therapies. A good example is rituximab, which binds to the CD20 protein on B cells and some cancer cells, triggering the immune system to eliminate them. (Institute, 2014, 2019b). Another example is blinatumomab, which binds to the proteins CD3 on the surface of T cells, and CD19, a protein present on the surface of leukemia cells. (Institute, 2017; Mocquot et al., 2022). This process helps the T cells get close enough to the leukemia cells to respond to and kill them.

Despite the promising results, CAR T-cell therapy has very high costs, has not shown long-term survival, and even T-cell cancers occur after treatment with CAR-T products (Dagar et al., 2023; Kandra et al., 2022; Sterner & Sterner, 2021). In the case of monoclonal antibodies, medical professionals are unable to predict the timing, severity, or adverse effects that may arise. Additionally, these antibodies have the potential to induce cytokine release syndrome and capillary leak syndrome, which can result in multiple organ failure and shock (Hansel et al., 2010; Izzedine et al., 2022). Conversely, small-molecule targeted drugs offer advantages in several areas, including pharmacokinetic (PK) characteristics, oral bioavailability, expense, patient adherence, storage, and transportation; additionally, they can target intracellular proteins, have the potential to achieve greater efficacy, and are generally safe when used to treat cancer (Liu et al., 2022; Sun et al., 2021; Zhong et al., 2021). Despite being challenged by toxicity, off-target effects, low response rates, and drug resistance, small-molecule targeted drugs still gain significant development (Lin et al., 2019; Liu et al., 2022; Zhong et al., 2021). There are currently 89 anti-cancer small compounds approved in China and the United States, with an average of 4 new small molecule medicines being released annually (Zhong et al., 2021).

However, challenges persist with tumor heterogeneity and acquired resistance. Tumor heterogeneity and acquired resistance pose significant challenges for targeted therapy in cancer treatment. Tumor heterogeneity refers to different genetic and phenotypic characteristics within a tumor. Because of this heterogeneity, treatment-resistant cancer cell subpopulations can occur, making it challenging to remove the tumor (El-Sayes et al., 2021; Lim & Ma, 2019; Lovly et al., 2016). Acquired resistance, on the other hand, results from pre-existing intratumor heterogeneity and the ongoing diversification of tumor cell phenotypes during treatment. This enables some tumor cells to survive the initial treatment and develop resistance to the targeted therapy, leading to disease relapse (Marusyk et al., 2020). The strategy to counter the situation is designing a personalized combinatorial application of

immunotherapy with targeted therapy. Monoclonal antibodies attach to the receptor and act as a signal for the immune cell to destroy the cancer cell, whereas small molecules enter the cell and inhibit the function of the key protein (Vanneman & Dranoff, 2012; Yu et al., 2019). Its efficacy can be increased using immune checkpoint inhibitors and radiotherapy (Yu et al., 2022). Now, NGS is also combined to provide genetic data so that personalized therapy can circumvent tumor heterogeneity and resistance issues (Avila & Meric-Bernstam, 2019; Nagahashi et al., 2019). It is a very active field of study that is evolving quickly as new therapies and drugs emerge.

Kinases, essential in cellular processes, regulate signaling pathways critical for growth, differentiation, and apoptosis. In cancer, dysregulated kinase activity drives uncontrolled proliferation, evasion of apoptosis, angiogenesis, and metastasis (Plati et al., 2008). Mutations or aberrant activation of kinases, such as EGFR and HER2, perpetuate tumorigenesis by sustaining growth and survival signals (Bou Antoun & Chioni, 2023; Wang et al., 2006). Targeting kinases in cancer therapy offers specificity and reduced toxicity compared to conventional chemotherapy. This success is exemplified by TKIs like imatinib, which revolutionized chronic myeloid leukemia treatment (Cohen et al., 2021; Kennedy & Hobbs, 2018). Kinase inhibitors comprise diverse compounds that target dysregulated signaling pathways prevalent in cancer. They are categorized into several types based on their mechanism of action and structural interactions with the targeted kinase. Type I inhibitors bind to the active conformation of the kinase, competing with ATP, while Type II inhibitors target the inactive conformation, exploiting adjacent hydrophobic pockets. Type III inhibitors form irreversible covalent bonds with cysteine residues, while allosteric inhibitors modulate kinase activity by binding to allosteric sites (Cohen et al., 2021; Martinez et al., 2020; Roskoski, 2016). However, despite these successes, resistance mechanisms, off-target effects, and signaling network redundancies pose challenges. Recent advances focus on overcoming resistance, yet limitations persist, urging exploration beyond kinase targets to address drug resistance, off-target effects, and signaling network complexity (Barouch-Bentov & Sauer, 2011; Bhullar et al., 2018).

Despite lacking catalytic activity, Pseudokinases serve crucial regulatory roles in signaling pathways and protein-protein interactions, highlighting their significance in cancer biology. Targeting pseudokinases represents a novel approach in cancer therapy, leveraging their unique structural features and regulatory functions to disrupt aberrant signaling networks driving tumor growth and metastasis (Sheetz & Lemmon, 2022; Sheetz et al., 2020). As researchers delve deeper into pseudokinase biology and develop more potent inhibitors, personalized medicine strategies tailored to individual patient profiles may optimize treatment efficacy while minimizing off-target effects. Integrating pseudokinase-targeted therapies into standard cancer treatment regimens signifies a

paradigm shift towards precision oncology, where innovative approaches harness the potential of pseudokinases as crucial and promising targets for combating cancer (Kung & Jura, 2019; Riegel et al., 2022).

ROR1, a receptor tyrosine kinase (RTK) family member, functions crucially in cancer progression despite lacking intrinsic catalytic activity (Borcherding et al., 2014; Zhao et al., 2021). Structurally, ROR1 comprises extracellular cysteine-rich and kringle domains, a transmembrane domain, and an intracellular tyrosine kinase-like domain devoid of crucial catalytic residues (Zhao et al., 2021). Its involvement in various cellular processes, including development and migration, underscores its multifaceted functions in cancer biology. Enhanced ROR1 expression, observed in leukemia, breast, lung, and ovarian cancers, correlates with aggressive tumor phenotypes and adverse clinical outcomes (Kipps, 2022; Saleh et al., 2019).

The pathways associated with ROR1 have been extensively discussed above. In summary, Wnt5a stimulates ROR1 to initiate signaling, activating multiple pathways supporting cancer cells. Although ROR1 typically forms a heterodimer with ROR2, this interaction isn't necessary for signaling. ROR2's intracellular domain activates ARHGEF1/2/6, promoting proliferation and migration. Additionally, interactions with 14-3-3ζ, DOCK2, HS1/cortactin, PI3K, Lyn, and HSP90 contribute to various cellular processes crucial for cancer progression, including survival, anti-apoptotic responses, and chemotaxis. This intricate network underscores the diverse role of ROR1 signaling in cancer physiology (Kipps, 2022; Mouawad et al., 2024).

The relationship between ROR1 and its family member, ROR2, is significant in cancer biology, as they share structural similarities and can heterodimerize, potentially intensifying downstream signaling effects (Rebagay et al., 2012; Sheetz et al., 2020). Targeting ROR1 for cancer therapy has gained attention, with monoclonal antibodies like cirmtuzumab disrupting ROR1-mediated signaling by impeding ligand binding and downstream pathway activation (Choi et al., 2018). Conversely, small molecule inhibitors like KAN0439834 inhibit ROR1 kinase activity and downstream signaling cascades, offering alternative therapeutic avenues (Daneshmanesh et al., 2018).

However, challenges persist in targeting ROR1 for cancer therapy, including potential off-target effects, acquired resistance, and the need to understand ROR1-mediated signaling networks further. Nonetheless, ongoing research aims to elucidate the intricacies of ROR1 biology and develop innovative therapeutic strategies to exploit its role in cancer progression. Finding small molecule inhibitors for ROR1 is a pressing need of the hour, representing a frontier in cancer research and a promising avenue for precision oncology. (Daneshmanesh et al., 2018; Fan et al., 2023; Fultang et al., 2019; Ghaderi et al., 2022; Gupta et al., 2023; Liu et al., 2019).

3. Research Gaps

Monoclonal antibodies (mAbs) targeting the ROR1 protein, exhibit limited efficacy in completely halting ROR1 functionality. They face challenges such as dependence on the intracellular domain for signaling and the extracellular domain for cytotoxicity, leading to resistance development. Additionally, these antibodies fail to induce immunological memory, have a short half-life necessitating repeated injections, and show resistance due to mutated tumor cells. Furthermore, they are ineffective against intracellular targets and depend heavily on high expression levels of the target antigen on tumor cell surfaces.

Efforts to target the ROR1 protein have encountered several gaps and challenges. The lack of disclosed drug structures and evidence of specific binding to ROR1's pseudo-kinase domain hampers the design of effective inhibitors. Additionally, comparisons with homologs like ROR2 are absent, which is crucial for understanding selectivity. While kinome analysis suggests structural similarities with CDK4, 5, 6, and 7, there is limited clarity on the specificity of compounds towards ROR1 relative to these kinases. The lack of toxicity studies further clouds safety evaluations, and the limited scope of testing across various cell lines and conditions limits the understanding of ROR1 inhibition's broader applicability.

4. Problem Statement

Continuous efforts are needed to target ROR1 protein, which play a crucial role in life threatening diseases. One of the most promising strategies is to find a core drug molecule that is highly specific and effective against ROR1. This core molecule can serve as a foundation for designing new inhibitors that can modulate ROR1 activity, contributing to more precise therapeutic approaches and potentially aiding in the development of personalized treatments.

5. Research Objectives

The objectives of the proposed study are as follows:

5.1. To identify ROR1 protein inhibitors using docking, shape screening, and MMGBSA analysis.

5.2. To select stable ROR1 inhibitors via replicated and extended molecular dynamic simulations.

5.3. To determine the binding energy of ROR1 protein inhibitors using Well-Tempered Metadynamics (WTM) and Absolute Binding Free Energy Perturbation (ABFEP).

6. Research Methodology

The proposed methodology is as follows:

6.1. To Identify ROR1 Protein Inhibitors Using Docking, Shape Screening, and MMGBSA Analysis.

To perform initial screening, a comprehensive compound library of more than 40 lakh chemical compounds will be sourced from open-access databases like PubChem and ZINC, along with commercial repositories such as Asinex, Selleckchem, Enamine, Maybridge, and ChemSpace. This extensive collection will enhance the exploration of potential drug candidates. Simultaneously, the 3D structure of the ROR1 protein, identified by its PDB ID: 6TU9, will be downloaded from the PDB database. Computational techniques will then predict interactions between the compounds and the ROR1 protein. A virtual screening pipeline will be applied, starting with High Throughput Virtual Screening (HTVS) docking, followed by Standard Precision (SP) and eXtra Precision (XP) docking for the top 10% of selected compounds. Novel compounds, identified through shape screening with at least 50% dissimilarity from known compounds, will undergo MMGBSA analysis. Compounds yielding an MMGBSA score equal to or below that of the bound ligand will be shortlisted as potential drug candidates.

6.2. To Select Stable ROR1 Inhibitors via Replicated and Extended Molecular Dynamic Simulations.

MD simulations will be conducted lasting 100 nanoseconds (ns) to assess stability, flexibility, and dynamic behavior. Compounds demonstrating the least fluctuations and stable binding interactions will be further validated. To validate the MD findings, five replicated simulations using random seeds will be performed to account for initial condition variability. Fluctuation values will be geometrically averaged and compared with selected compounds. Compounds displaying minimal fluctuations will be subjected to extended simulations lasting 1000 ns (1 microsecond) to explore conformational space and dynamics.

6.3. To Determine the Binding Energy of ROR1 Protein Inhibitors Using Well-Tempered Metadynamics (WTM) and Absolute Binding Free Energy Perturbation (ABFEP).

Metadynamics will be employed to quantify the strength of interaction between the drug candidate and its target, using ten windows of 10 ns each with random seeds (velocities), a temperature of 310K, 0.5 height, 25Å wall, and a 3.4 kTemp bias factor. Binding site residues and ligands will serve as collective variables, and the resulting data will be averaged to calculate the Dissociation Free Energy (DFE) using a Python script. This analysis will provide insights into the binding pathway, affinity,

and stability by estimating the energy required to dissociate the protein-ligand complex. In parallel, sophisticated computational techniques such as Absolute Binding Free Energy Perturbation (ABFEP) calculations, using Thermodynamic Integration (TI), will be conducted via the BFEE2 tool with NAMD. This approach will systematically transform the drug candidate from a non-bonded to a fully bound state within the protein's binding site. By estimating free energy differences across intermediate states, this method will accurately assess binding affinity, considering both enthalpic and entropic contributions. Together, these computational analyses will be critical for prioritizing lead compounds with the highest potential for success in subsequent drug development stages.

7. Expected Outcome of the Research

Identifying potential lead compounds capable of strong and stable binding with the pseudokinase domain of the ROR1 protein. The study could leverage advanced in-silico methods such as shape screening, replicated and extended simulations, well-tempered metadynamics, and absolute binding free energy calculations to find suitable leads. This initial phase would allow for efficient screening of compound libraries and help establish the foundation for further investigation into the effectiveness of the leads. Although the process is challenging, each step could bring us closer to the goal of discovering novel and effective ROR1 inhibitors.

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Summary

Computational Identification of Small Molecule Inhibitors to Target ROR1 Protein

SHORT SYNOPSIS

by

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22-23/Ph.D/W/016

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Abstract

The ROR1 protein has emerged as a highly promising therapeutic target due to its involvement in critical signaling pathways that is linked to severe pathological conditions. Current treatment strategies, including monoclonal antibodies, antibody-drug conjugates, and immunotoxins, have demonstrated considerable potential in targeting ROR1, but their clinical efficacy is often limited by significant drawbacks, such as immune-related adverse effects, off-target toxicity, and the eventual emergence of therapeutic resistance. To address these challenges and improve therapeutic outcomes, this study introduces a comprehensive *in-silico* research methodology that employs

advanced computational techniques, including shape screening, well-tempered metadynamics, and alchemical binding free energy calculations. These approaches are applied to systematically identify, evaluate, and optimize lead compounds with a strong and stable binding affinity for the pseudokinase domain of ROR1 protein. By focusing on this domain, the study aims to discover novel, ROR1 specific inhibitors that offer enhanced efficacy, improved safety profiles, and a reduced risk of resistance development, thereby significantly contributing to the advancement of next-generation, targeted therapies for ROR1 associated diseases.

Keywords: Cancer, Drug Discovery, Inhibitor, In-silico, Kinase, Pseudokinases, ROR1, Small Molecules

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